REMARKS

Status of the Claims

Claims 14-17, 19, 20, 22 and 23 are pending in this application. Claims 21 and 24-26 have been canceled. No claims have been added. The claims have been amended to recite that the bacteria is enteric bacteria having fimbriae. Support is found at page 2, line 5. Claim 1 has also been amended to recite that the growth phase is a logarithmic growth phase. Support is found at page 5, line 18. No new matter has been added by the above claim amendments.

Rejections under 35 USC 112, second paragraph

The Examiner rejects claims 14-17 and 19-26 as indefinite. The Examiner specifically rejects claim 14 for the phrase "detecting antigens which are expressed soon after inoculation" because the time period is unclear. Applicant amends claim 14 to delete the phrase "soon after inoculation". As such, the rejection should be withdrawn.

The Examiner rejects claim 14 for the phrase "before an actual growth phase." The Examiner also rejects claim 14 for the phrase "in the beginning of the growth phase." Applicant amends claim 14 to insert that this phrase is the "logarithmic" growth phase. Applicants also submit a definition of logarithmic growth phase as

defined in Dictionary of Microbiology and Molecular Biology as Exhibit B. As such, the rejections should be withdrawn.

The Examiner also rejects claims 15, 16, 19, 20 and 24-25 as indefinite. The Examiner rejects claims 15 and 16 for the phrase "wherein bacterial antigens are detected". Applicant amends claim 15 to recite "fimbrial antigens" instead of "bacterial antigens". As such, this rejection should be withdrawn.

Claim 20 is also rejected for reciting "microbial" antigens. Applicant amends claim 20 to recite "fimbrial" antigens. As such, this rejection should be withdrawn.

Claim 19 is rejected for no antecedent basis for "fimbrial proteins". Applicant amends claim 19 to recite "fimbrial" antigens, which has antecedent basis in claim 14. Claim 19 is also rejected for the phase "or comparable to them". This phrase has been deleted. As such, these rejections should be withdrawn.

Claims 24 and 25 are rejected as indefinite. Applicant cancels these claims; thus, the rejections are moot and should be withdrawn.

Rejections under 35 USC 112, first paragraph

The Examiner rejects claims 14-17 and 19-26 as not enabled for all bacteria having fimbriae and for derivatives of SEQ ID NO: 1. Applicant traverses the rejections and respectfully requests the withdrawal thereof.

Applicant amends the claims to define the bacteria having fimbriae as enteric bacteria having fimbriae. This subgenus of bacteria having fimbriae is commensurate in scope with the disclosure. This subgenus is defined and supported by the specification. Salmonella bacteria is a representative species within the subgenus of enteric bacteria having fimbriae. Clearly, Salmonella is supported and enabled by the specification. Applicant also submits that the entire subgenera of enteric bacteria having fimbriae is supported and enabled by the specification.

It is well known that Salmonella are members of enteric bacteria as Salmonella attach to the gut epithelium of the host by fimbriae. This is how Salmonella become pathogenic to the host. *Enterobaceriaceae* are classified as such for this ability to attach to the gut epithelium with fimbriae.

Applicant has conducted further experiments with *Citrobacter* and *Klebsiella*, two other bacteria within the subgenus of enteric bacteria having fimbriae. The results of the experiments are shown in Figures 1 and 2 attached hereto as Exhibit A. The figures demonstrate that the immunoreactivities of strains of *Citrobacter* and *Klebsiella* arose in 4 hours, whereas the peak occurred in 5 hours. In view of this data, the earlier onset of fimbrial expression seems to be valid irrespective of the specific species within the subgenus of enteric bacteria having fimbriae. Applicants also submits that there is no undue experimentation in determining

which bacteria are enteric bacteria having fimbriae. The family of *Enterobacteriaceae* is well known and Applicant has demonstrated with Figures 1 and 2 (Exhibit A) that other members of the family *Enterobacteriaceae* are detectable with the claimed method.

Please find enclosed herewith Exhibit C, a manuscript which was a part of the Academic Dissertation of the Inventor (E. Hakalehto: Characterization of *Pectinatus cerevisiiphilus* and *P. frisingiensis* Surface Components. Use of Synthetic Peptides in the Detection of Some Gram-negative Bacteria. University of Kuopio Publications C. Natural and Environmental Sciences 112.2000).

In the thesis, the same finding was clearly indicated as in the priority application of this application. The amount of detectable fimbrial antigens surprisingly peaked only after a few hours of cultivation, in the very beginning of the exponential growth phase surprisingly regardless of the cell number.

As such, Applicant submits that the claims are enabled for "enteric bacteria having fimbriae" and the rejection regarding the scope of the bacteria should be withdrawn.

Regarding claim 20, Applicant amends claim 20 to delete the phrase "or a derivative thereof." As such, this rejection should also be withdrawn.

Rejection under 35 USC 103(a)

The Examiner rejects claims 14-17 and 19-26 as obvious over Thorns et al. USP 5,510,241 (Thorns '241) in view of Blakburn. Applicant traverses the rejection and respectfully requests the withdrawal thereof.

Applicant submits that Thorns '241 discloses a method of testing for the presence of Salmonella by detecting antibodies that are specific to Salmonella. Thorns '241 fails to disclose or suggest a fast detection assay where the presence of Salmonella is detectable within 3 to 10 hours as with the present invention.

The Examiner relies on Blakburn for disclosing a method of shorting the time for detecting bacteria. However, Applicant submits that Blakburn merely discloses a method of shortening the selective enrichment steps. Blakburn fails to disclose a method of early detection of enteric bacteria having fimbriae in the early phases of growth. Blakburn states in the last sentence of the article "the application of separation and concentration techniques, together with different approaches to pre-enrichment to prevent competitive inhibition of salmonella, should improve the reliability of salmonella testing and reduce the length of cultural enrichment." Clearly, Blakburn is primarily concerned with shortening the length of cultural enrichment.

As such, Applicant submits that the combination of teachings from Thorn '241 and Blakburn do not motivate one of ordinary skill

in the art to make the method of the present invention, because the secondary reference Blakburn is deficient and does not teach the same mechanism for early detection. Therefore, Applicant respectfully requests that this rejection be withdrawn.

Conclusion

As Applicants have addressed and overcome all rejections in the Office Action, Applicants respectfully request that the rejections be withdrawn and that the claims be allowed.

Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicant(s) respectfully petition(s) for a three (3) month extension of time for filing a reply in connection with the present application, and the required fee of $475.00 is attached hereto.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Kecia Reynolds (Reg. No. 47,021) at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

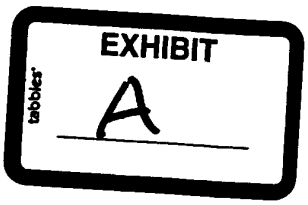
By _____
Gerald M. Murphy, Jr., #28,977

GMM/RJR/crt
0933-0162P

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

Attachment(s): Exhibits A, B and C

(Rev. 09/30/03)



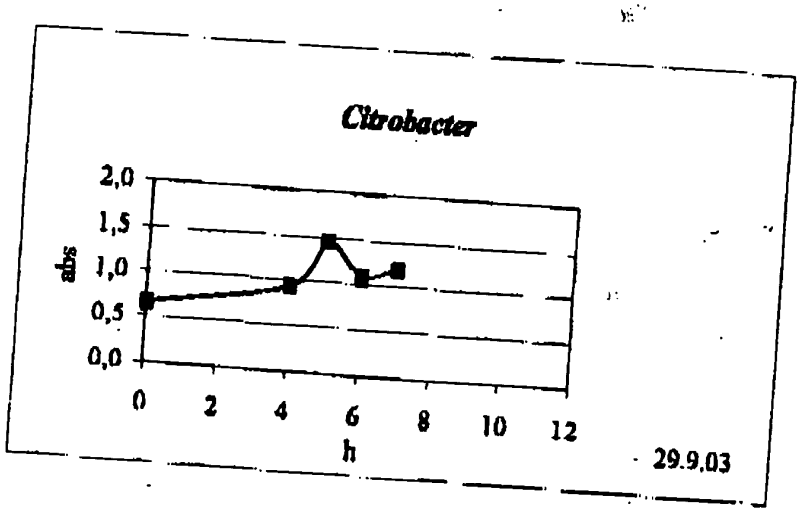


Figure 1

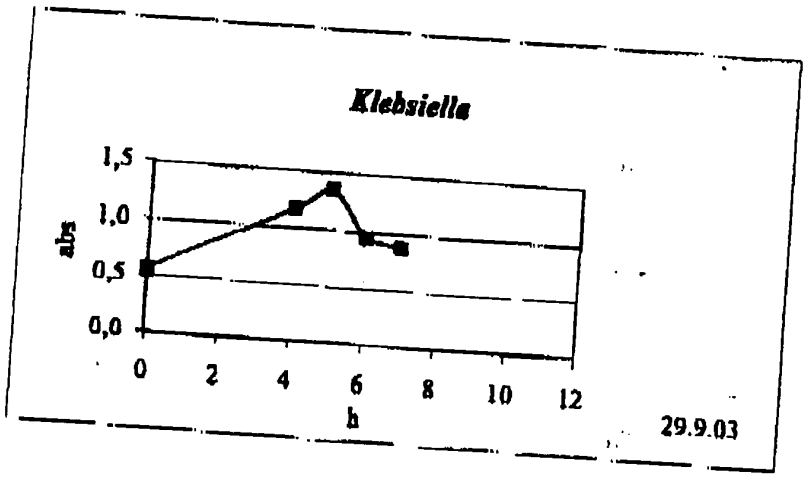


Figure 2

Rise in immunoreactivities of *Citrobacter freundii* (Figure 1) and *Klebsiella* sp. (Figure 2) strains from enrichment cultures studied with an anti-peptide antibody against entero-bacterial type-1 and SEF fimbrial sequences (EIA-assay). The rise in activities could be measured in 4 hours, the peak occurring at 5 hours from the onset of the enrichment cultivations.

Singleton and Sainsbury:
Dictionary of Microbiology and Molecular Biology
John Wiley & Sons

erally. Rust fungi characteristically form four basidiospores per basidium; in many smut fungi the basidium may give rise to an indefinite number of basidiospores following mitotic divisions within the metabasidium. [Review: BBMS (1983) *17* 82–94.]

basionym (basonym) The name of the species whose specific epithet is included in a new combination (see COMB. NOV.).

basipetal development Development from the tip towards the base or point of attachment; e.g., in a chain of basipetally developing spores the first-formed spores occupy positions in the terminal or distal parts of the chain, while spores formed later occupy the more proximal positions. (cf. ACROPETAL DEVELOPMENT.)

basonym *Syn.* BASIONYM.

basophil A PMN (q.v.) which can respond to certain stimuli e.g. by rapidly secreting vasoactive products; it is primarily a circulatory cell, and is important e.g. in immediate-type hypersensitivity reactions. As in the MAST CELL, with which it shares many characteristics, the basophil surface contains e.g. many high-affinity receptor sites for the Fc portion of IgE antibodies, and its (basophilic) cytoplasmic granules also contain substances such as HISTAMINE and SEROTONIN; stimuli which cause activation and degranulation lead to the secretion of various products and to the formation of e.g. SUPEROXIDE and H_2O_2. (See also JONES–MOTE SENSITIVITY.)

batch culture (closed culture) A form of CULTURE (sense 2) in which a given volume of liquid medium is inoculated with cells (e.g. bacteria) capable of growth in that medium, and the inoculated medium is incubated for an appropriate period of time; cells growing under these conditions are exposed to a continually changing environment due e.g. to the gradual consumption of nutrients and the accumulation of metabolic wastes (cf. CONTINUOUS CULTURE and FED BATCH CULTURE).

The growth curve (see GROWTH) obtained by monitoring a batch culture commonly exhibits a sequence of four main phases of growth. In the *lag phase* the growth rate — i.e., the rate of increase in cell numbers (or biomass) — is initially minimal but subsequently rises to a value dictated e.g. by the prevailing conditions (e.g. temperature, concentration of nutrients etc). The length of the lag phase is influenced by the cultural history of the cells in the inoculum. For example, if slowly dividing cells from a nutrient-poor environment are transferred to a nutrient-rich medium which can support a higher rate of growth, there is usually a relatively long lag phase during which time the cells become adapted to the new environment; during this period of adaptation the cells exhibit *unbalanced* GROWTH. Subsequently, growth occurs at a new, higher rate permitted by the higher levels of nutrients. A long lag phase may also occur e.g. if the carbon source in the new medium differs from that previously used by the cells. (cf. DIAUXIE.) When actively dividing cells are transferred to a medium which offers conditions similar to those under which the cells were previously growing, a lag phase is not observed.

At the end of the lag phase the cells enter the *exponential* (= *logarithmic* or *log*) *phase* of growth in which, for a given organism, the growth rate is both constant and maximal for the particular growth conditions. In this phase there is an exponential increase in cell numbers (and biomass); this type of growth is referred to as *balanced* GROWTH. (See also TROPHOPHASE.)

In the *stationary phase* the growth rate declines and eventually reaches zero. (See also IDIOPHASE.)

In the *death phase* the number of viable cells in the culture (maximal in the stationary phase) declines.

batch retort In CANNING: a vessel within which filled, sealed cans (or other containers) undergo heat treatment in a batch-type process (cf. COOKER–COOLER). The cans are subjected to saturated steam under pressure or to an air–steam mixture, or (in an *overpressure retort*) are submerged in heated water under an air pressure of up to ca. 250 kPa. In some batch retorts (e.g. the *Konservomat*) the load is agitated to facilitate heat penetration.

bating (of hides) See PROTEASES.

Batrachospermum See RHODOPHYTA.

Battarrea See GASTEROMYCETES (Tulostomatales).

Battey bacillus Strain(s) of *Mycobacterium intracellulare* or, loosely, strain(s) of related species (including *M. avium*).

Bayer's junction (Bayer's patch) See ADHESION SITE.

Bayleton See TRIADIMEFON.

BB-transhydrogenase See TRANSHYDROGENASE.

BCDF (*immunol.*) See LYMPHOKINES.

BCF Bioconcentration factor: a measure of the degree to which a compound (commonly a xenobiotic), present in an aquatic environment, is accumulated in the biomass of organisms (e.g. algae) living in that environment: $BCF = C_o/C_w$, where C_o = concentration of the compound in the organisms, and C_w is the concentration of the compound in the water. (See e.g. TBTO.)

BCG Bacillus (or bacille) Calmette–Guérin: an attenuated strain of *Mycobacterium bovis* which is used e.g. as a vaccine against tuberculosis. The administration of BCG can also

Kuopion yliopiston julkaisuja C. Luonnontieteet ja ympäristötieteet 112
Kuopio University Publications C. Natural and Environmental Sciences 112

Elias Hakalehto

Characterization of *Pectinatus cerevisiiphilus* and *P. frisingiensis* Surface Components

Use of Synthetic Peptides in the Detection of Some Gram-negative Bacteria

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium L23, Snellmania building, University of Kuopio, on Thursday 8th June 2000, at 12 noon

Institute of Applied Biotechnology
University of Kuopio

Kuopio 2000

GROWTH-PHASE LIMITED EXPRESSION AND IMMUNOLOGICAL DETECTION OF *SALMONELLA* TYPE 1 FIMBRIAE

Elias Hakalehto[1*], Helena Hujakka[2], Sanna Airaksinen[3], Jari Ratilainen[2] and Ale Närvänen[2]

1) Institute of Applied Biotechnology, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland

2) Department of Chemistry, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland

3) Equine Information Centre, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland

* corresponding author, tel. + 358-17-162211, fax. + 358-17-163148

Abstract

Fimbrial expression was examined using antibodies to *Salmonella enterica* type 1 fimbrin peptides. Peak immunoreactivity occurred after 3 h of cultivation at 42 °C and after 9 h at 20 °C in shaken cultures. In electron micrographs, the highest numbers of fimbriae occurred at the time of peak immunoreactivity, which suggests that fimbriae were assembled during a short period of the early exponential growth phase.

Once entering the human body salmonellas rapidly invade the gut epithelium. They possess several surface structures, fimbriae, for attachment. The most intensively studied fimbrial structures are the enterobacterial type 1 fimbriae, which mediate the mannose-sensitive (MS) binding of the bacterial cells to the target cells (12). Type 1 fimbriae are consisting of filament proteins, fimbrins, being straigth rod-like structures of about 1 µm in length and 7 nm thick. The fimbrins are synthesized at the cytoplasmic membrane and directed to the periplasmic space, where chaperones readily bind to them (8). The chaperones liberate the fimbrin molecules added to the growing chain of the fimbrial filament. The fimbrial assembly occurs about 3 minutes after the synthesis of fimbrial subunits, and the reservoir of the synthesized fimbrins is limited in number (3). On the other hand, the fimbrial assembly is suggested to occur without protein synthesis. In static cultures the fimbriation has been suggested to offer a selective advantage for the fimbriated cells by allowing them to access oxygen on the broth surface by forming a mesh of cells (13).

The type 1 fimbriae of *Escherichia coli*, *Salmonella* sp. and other enteric bacteria are important mediators of bacterial adhesion and invasion (6). Similar structures have been found in other bacteria, such as *Pseudomonas aeruginosa* (18). Based on physiological or environmental condition bacterial cells vary between fimbriate and nonfimbriate state. They are also subject to phase variation which is under transcriptional control (4, 19). In the present study we have studied the expression of type 1 fimbriae on *Salmonella* cells using anti-peptide antibodies to the fimbrin protein with enzyme immuno assay and transmission electron microscopy. The fimbriae were monitored on intact *Salmonella enterica* serovar Typhimurium and serovar Enteritidis cells at two different temperatures.

The synthetic peptide. The amino acid sequence for the synthetic peptide was derived from

Salmonella enterica serovar Typhi type 1 fimbrin (17). The selected sequence ASIFTAIGDTTAQVPPSIV shares 52.9 % identity in 17 as with corresponding polypeptide of *E. coli* fimbrin type 1 (1) (Sequence similarity and homology program Fasta3, EMBL). A peptide was synthesized as multiple-antigen peptide (MAP) (19) with four branches using Millipore's PerSeptive 9050 Plus automated peptide synthesizer and Fmoc synthesis strategy. Fmoc-Lys(Fmoc)-OH comprised the backbone of the branched structure. The branched peptide was used for immunization without conjugation to carriers. Rabbits were subcutaneously immunized with 500 µg of MAP-peptide in Freund's complete adjuvant. Boosters (500 µg) were injected in Freund's incomplete adjuvant every two weeks, for five months.

Bacterial strains and culture conditions. The strain *Salmonella* serovar Enteritidis phagetype 4 (IHS 59813) and the *Salmonella* serovar Typhimurium phagetype 1 (IHS 59929) were stored at 37 °C in THG medium (5 % tryptone, 2.5 % yeast extract, 1 % glucose) and seeded every two weeks troughout the study. Cultivation was started with 3-4 days old starter cultures by inoculating 5 % of the cultures into fresh RVS medium (Rappaport-Vassiliadis soya peptone broth, Oxoid, UK). The cultures were shaken in Erlenmeyer flasks (100 ml each) at 20 and 42 °C. Samples were stored at 4 °C before coating to microtitration plates, up to 8 hours after cultivation at 42 °C and up to 24 hours after cultivation at 20 °C. The cell densities were determined by plate culture. Transmission electron microscopy (TEM) were taken at 0, 3.5 and 7 hours. For the plate cultivation samples were diluted to the dilutions 10^{-2}-10^{-8} with 0.9 % NaCl, added to XLD-plates (xylose-lysine-deoxycholate) and incubated at 37 °C for 24 hours. The number of colonies were counted and colony forming units (cfu) / ml were calculated for every time point.

uence
ptide
L). A
sing
basis
The
were
ent.
five

Enzyme immunoassay (EIA). The reactivity of the fimbrial anti-peptide antibodies with whole cells of *Salmonella* serovar Typhimurium was tested with conventional indirect EIA (5). Microtitration plates (MicroTest III, Becton & Dickinson) were first pre-treated with 0.5 % glutaraldehyde. The bacterial coating of the microtiter wells was adapted to the same level in different samples, 1:2 dilution of 0 time point as reference. Microtitration wells were coated by bacterial cells (1-2 x 10^6 cfu), incubated overnight at 4 °C and tested with rabbit anti-peptide antibodies (1:100). Bound antibodies were detected with alkaline phosphatase conjugated anti-rabbit IgG and visualised with para-nitrophenyl.

The reactivity of the fimbrial anti-peptide antibodies with purified fimbriae was tested with recombinant *Salmonella* serovar Typhimurium type 1 fimbriae isolated from *E. coli* (pISF101) (17), and with *E. coli* type 1 fimbriae purified from LE392/pRPO-1 strain (16). The EIA for purified fimbria was carried out on Maxisorp Nunc-immuno plate coated with isolated fimbriae in TBS. The plates were not pretreated and fimbriae were exponentially titrated from 10 µg/ml. Otherwise, the plates were treated as in the EIA-procedure for whole cells.

Transmission electron microscopy (TEM). The grids were first carbon-coated (Vacuum evaporator, JEOL JEE 4 B, Tokyo, Japan) and radiated overnight with UV-light. Immediately after sampling, 10 µl of the growth suspension was pipetted on the grid, incubated for 10 min and dried with blotting paper moistened with water. Grids were stained with 0.5 % or 1 % phosphotungstic acid (PTA) (Merck) or 1 % sodiumsilicotungstate (Sigma) in water for 15 to 30 seconds and dried with blotting paper. Grids were viewed with JEOL JEM-1200 EX electron microscopy.

Anti-peptide antibodies reacted in EIA both with recombinant *Salmonella* serovar Typhimurium type 1 fimbriae and with *E. coli* type 1 fimbriae purified from LE392/pRPO-1 strain. The detection limit of the assay varied between 1 and 5 µg/ml. The sequence similarity was high enough to produce antibodies cross-reacting with these two type 1 fimbrial variants. Therefore, the produced antibodies could be considered as anti- type 1 fimbrin antibodies.

The maximum reactivity of anti-peptide antibodies with bacterial cells cultivated at 42 °C was reached at 3 hours, after which the reactivity rapidly decreased (Fig. 1A). The low growth yield was apparently due to the combination of relatively high growth temperature and the selective growth medium. At 20 °C there was already immunoreactivity at the starting point and the maximum reactivity was reached at 9 h (Fig. 1B). There was essentially no EIA reactivity after 9 h. The cfu was 2×10^7 at the starting point, 5×10^7 at 9 h and 40×10^7 at 24 h. The cell growth did not reach the stationary phase during the 24 h cultivation. When bacteria were grown at 20 °C for 24 h and the suspension was transfered to 42 °C and cultivated for 8 hours, the bacteria did not seem to express fimbrin on the cell surface in spite of exponential growth. This indicated that the onset of fimbrin synthesis is dependent on other factors than correct temperature or being in the active growth phase, only. As no pellicle was formed in shaken cultures, the formation of fimbriae was not depending on pellicle formation. The results suggest that the fimbriae are transferred out of the bacterial cell only during a short period of the growth cycle. This suggested that the ceased *de novo* synthesis of fimbrial subunits during the growth at low temperature did not restart as a consequence of increased temperature only (Fig. 1C).

In the electron microscopy a clear pattern of fimbriation was observed at 3.5 hours of

culture at 42 °C (Fig 2A and 2B) whereas at the starting point (Fig 2C) and at 7 hours (Fig 2D) only few fimbriae could be seen. Most *Salmonella* cells were clearly smaller after five hours of cultivation, as compared with the same cells after 3-4 hours of growth in the RVS medium. It is noteworthy, that at the temperature shifts (from 18 °C and 37 °C to 42 °C) the peak of the immunoreaction occurred at the same period of time (at about 4 hours) when fresh medium was added (results not shown).

The elongation of bacterial fibrillar structures, such as flagellae or fimbriae, has been shown to take place in a few minutes (3). The rapid growth requires the building blocks, flagellin or fimbrin proteins, to be either synthetized quickly or to be stored in the cytoplasm or the periplasmic space. In the present study we have shown that under favourable conditions, *Salmonella* strains start expressing type 1 fimbriae before the onset of the logarithmic growth. This is in good agreement with the findings on the regulation of flagellar assembly of *Salmonella* serovar Typhimurium (10) and *Caulobacter crescentus* (10). In the latter organism, a membrane-associated protein, fliX, is a necessary element at an early stage of flagellar assembly. Expression of the fliX gene is under cell cycle control, being at the highest level in predivisional cells (10). Similar regulation could be possible also in the case of *Salmonella* fimbriae. Our findings are in good agreement with these of Dodd and Eisenstein (3), who observed that the synthesis of type 1 fimbrin proteins in *E. coli* started during the lag-phase and was responsible for up to 98 % of the total protein synthesis at that time point (about 1.5 hours after inoculation). The synthesis decreased to 20 % of the protein synthesis during the late logarithmic phase (280 minutes after inoculation). However, in this case the fimbriated cells were still present in high numbers, apparently due to the fact that the study was carried out in static cultures, which favour fimbriated cells (13).

The microbial growth in suspension cultures usually occurs in the form of an S-shaped curve, where the onset of the exponential growth phase is preceded by a lag-phase and induced apparently by the sudden changes in the growth conditions. The duration of the lag-phase is about 2 hours, and it is believed to be needed for the cells to adjust their metabolism as well as some other capabilities, such as motility and attachment properties, to the changed conditions of the culture. This phenomenon frequently takes place when the inoculum is transferred into fresh medium. It seems likely that the synthesis of type 1 fimbrin starting already during the stationary phase gives the bacterial cells some advantages in the new conditions.

Outside the gastrointestinal tract salmonellas are often under environmental stress, and cells have to cut their metabolic level to the minimum. In river water *Salmonella* serovar Typhimurium cells were found to survive as long as 35 days and in lake water 48 days (19). In sterile water, they were able to withstand nutrient deprivation for several months. When engulfed with food or drinking water, *Salmonella* cells have to rebuild their surface structures after the more or less demanding conditions of the acidic stomach. At the gastric pH gram-negative cells seem to loose readily their outer membranes as vesicles, and flagella and most likely other appendages are dissociated and dissolved into the gastric juice (7). Type 1 fimbriae are totally dissociated at pH 2.2 (12). The presentation of type 1 fimbriae before the onset of the most active growth could be related to the need for establishing means for the attachment of the cells into the gut epithelium.

It has been shown that in the static liquid cultures fimbriation gives a selective advantage to the fimbriated cells in comparison with non-fimbriated mutants (13). The fimbriae help the cells in constructing a mesh onto the liquid surface, giving them a constant access to oxygen.

In shaken flask cultures no such benefit is offered by fimbriation, and these conditions presumably reflect better the conditions *in vivo*. Under the conditions of the gastrointestinal tract, an early attachment could benefit the fimbriated cells in helping them to attach to the nutrient-rich regions of the gut epithelium and thus avoiding to get flushed away. On the other hand, the period of extensive type 1 fimbrial expression proved to be relatively short, and finished before the growth reached the stationary phase. This may correlate with the density of the bacterial culture in the intestine, where it might be advantageous to detach the cells and spread them out if the density becomes too high, and the availability of the nutrients decreases.

The genus *Salmonella* is a wide group of enteroinvasive and pathogenic gram-negative bacteria. Specific antibodies against *Salmonella* antigens are usually more or less serotype-specific, which makes the efforts for an overall immunological resolution of *Salmonella* strains complicated. On the other hand, many closely related enteric bacteria, such as *Citrobacter* sp., can cause false positive results in the immunodetection (15). Also the remarkable variation in the major antigenic molecules is complicating their detection by different assays. One possible explanation for the decreasing levels of type 1 fimbriae after exponential growth phase could be related to the finding that encapsulated *Klebsiella pneumoniae* strains did not possess type 1 fimbriae on their surfaces (9). In the case of *Salmonella* the alterations in the cell surface layers during the different growth phases could also somewhat block the assembly of the fimbriae or at least prevent their immunodetection to some extent.

Rapid screening of the *Salmonella* isolates can be accomplished by combining their immunological detection using antipeptide antibodies against type 1 fimbrial antigen with the

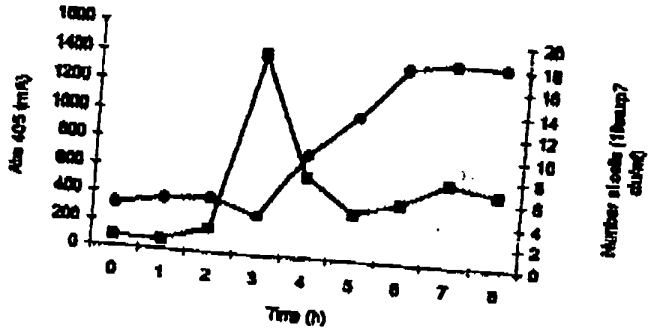
enrichment of this antigen. The prerequisite is that the bacteria produce type 1 fimbriae. The enzyme immunoassay employing synthetic peptide antibodies could also be useful for the rapid screening of salmonellas directly from various environmental, industrial or clinical samples after a short pre-enrichment cultivation, or as a preliminary check for samples prior to PCR or other further testing (2).

Acknowledgements

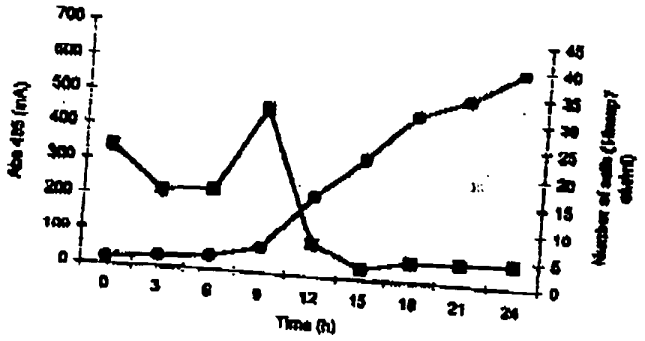
We thank Dr. Anja Siitonen (National Public Health Institute of Finland, Laboratory of Enteric Pathogens) for giving us the bacterial strains and for helpful suggestions, and Professor Timo K. Korhonen (Department of Biosciences, University of Helsinki, Finland) for providing us with the isolated fimbrin proteins. We also thank Professor Jukka Finne (Department of Medical Biochemistry, University of Turku, Finland) for constructive criticism. This work was funded by Finnoflag Oy, Kuopio, Finland.

Figure 1. The reactivity of anti-peptide antibodies with *S. typhimurium* cells tested with indirect EIA-method. The results are in milliabsorbance units. The binding of antibodies (- ■ -) was compared to the cell growth (- ● -).

1 A The binding of antibodies and the growth curve of the bacterial cells cultivated at 42 °C. The maximum reactivity is reached after three hour cultivation when the number of cells is $4x10^7$.



1 B The growth and reactivity of anti-peptide antibodies with bacterial fimbrin cultivated at 20 °C. The maximum reactivity is reached after nine hour cultivation when the number of cells is $4x10^7$.



1 C The growth and reactivity of anti-peptide antibodies with bacterial fimbrin cultivated at 42 °C after preculture at 20 °C for 24 hours. Essentially no fimbrin type 1 expression can be seen.

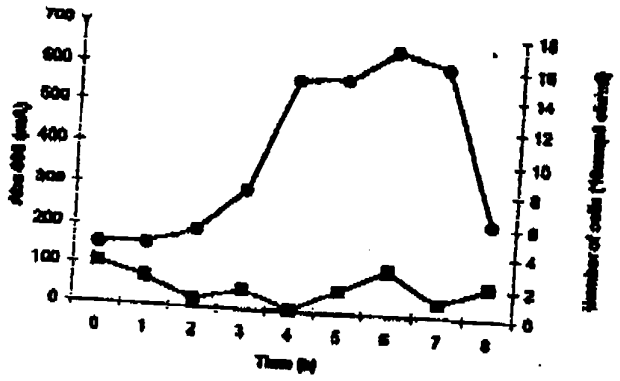
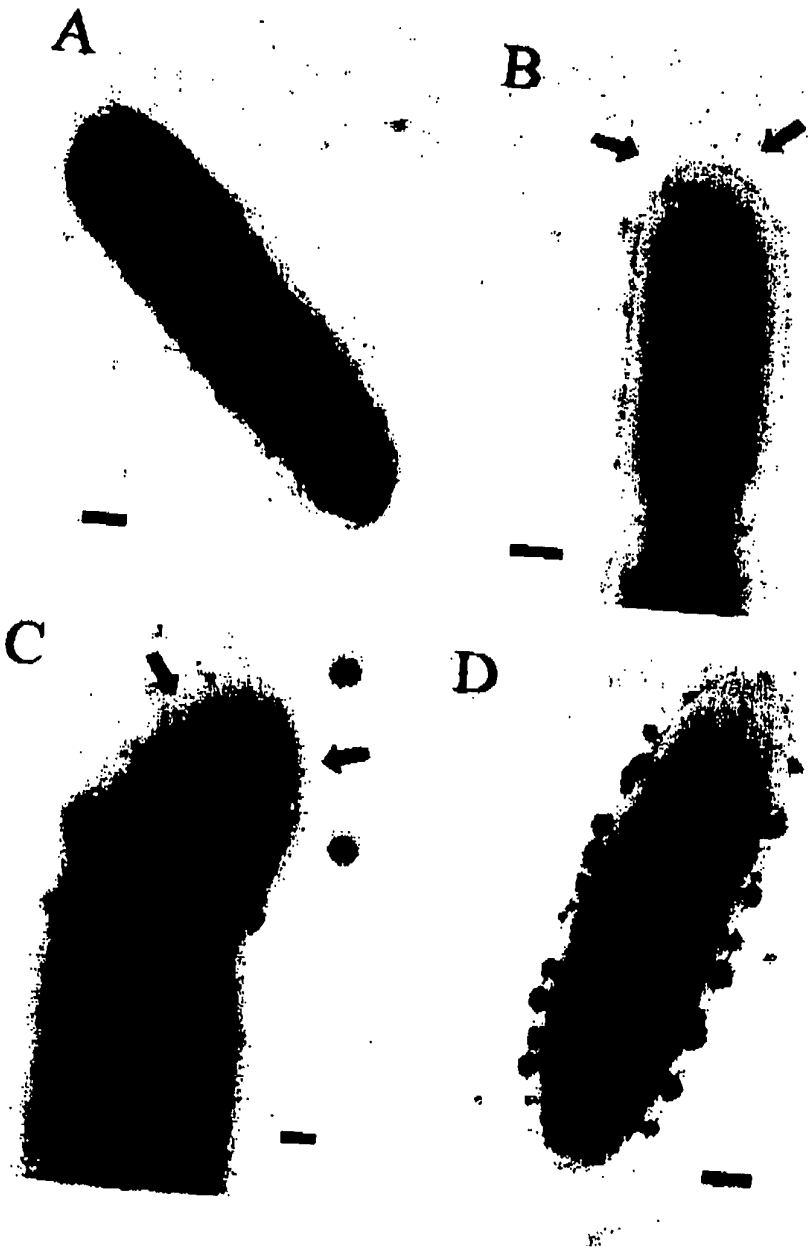


Figure 2. Transmission electron micrographs of *Salmonella* cells grown at 42 °C. *A*) a cell of the inoculum (after 0 hours of cultivation), stained with sodiumsilicowolframate; B) a cell in the early logarithmic phase (after 3.5 hours of cultivation), stained with sodiumsilicowolframate; *C*) same as *A*, but stained with phosphowolframic acid; and D) a cell in the late logarithmic phase (after 7 hours of cultivation), stained with phosphowolframic acid. Regardless of the staining method, the fimbriated cells appeared in high numbers in the early or mid-logarithmic growth phase. The bar in the micrographs represents 200 nm. (on the next page)

Figure 2.



References

1. Blattner, F. R., G. 3rd Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277:1453-74
2. Burtscher, C., P. A. Fall, P. A. Wilderer and S. Wuertz. 1999. Detection of *Salmonella* spp. and *Listeria monocytogenes* in suspended organic waste by nucleic acid extraction and PCR. Appl. Environ. Microbiol. 65:2235-2237.
3. Dodd, D. C. and B. I. Eisenstein. 1984. Kinetic analysis of the synthesis and assembly of type 1 fimbriae of *Escherichia coli*. J. Bacteriol. 160:227-232.
4. Eisenstein, B. I. 1981. Phase variation of type 1 fimbriae in *Escherichia coli* is under transcriptional control. Science 214:337-339.
5. Engvall, E. and A. J. Pesce. 1978. Quantitative enzyme immunoassay. Scand. J. Immunol. 8. Suppl. 7.
6. Ernst, R. K., D. M. Dombroski and J. M. Merrick. 1990. Anaerobiosis, type 1 fimbriae, and the growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. Infect. Immun. 58:2014-2016.
7. Hakalehto, E., A. Haikara, T.-M. Enari and K. Lounatmaa. 1984. Hydrochloric acid extractable protein patterns of *Pectinatus cerevisiiphilus* strains. Food Microbiol. 1:209-216.
8. Hultgren, J. J., J. Abraham, M. Caparon, P. Falk, J. W. St. Geme III and S. Normark. 1993. Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition. Review. Cell. 73:887-901.
9. Matatov, R., J. Goldhar, E. Skutelsky, I. Sechter, R. Perry, R. Podschun, H.

Sahly, K. Thankavel, S. N. Abraham and I. Ofek. 1999. Inability of encapsulated *Klebsiella pneumoniae* to assemble functional type 1 fimbriae on their surface. FEMS Microbiol. Lett. 179:123-130.

10. Mohr C. D., J. K. Mackichan and L. Shapiro. 1998. A membrane-associated protein, FliX, is required for an early step in *Caulobacter* flagellar assembly. J. Bacteriol. 180:2175-85.

11. Muramoto, K., S. Makishima, S.-I. Aizawa and R. M. Macnab. 1998. Effect of cellular level of FliK on flagellar hook and filament assembly in *Salmonella typhimurium*. 277:871-882

12. Müller, K.-H., S. K. Collinson, T. J. Trust and W. W. Kay. 1991. Type 1 fimbriae of *Salmonella enteritidis*. J. Bacteriol. 173:4765-4772.

13. Old, D. C. and J. P. Duguid. 1970. Selective outgrowth of fimbriated bacteria in static liquid medium. J. Microbiol. 163:447-456.

14. Pearson, R. 1990. Rapid and sensitive sequence comparison with FASTP and FASTA. Methods Enzymol. 183:63-98.

15. Popoff, M. Y. and L. Le Minor. 1997. Antigenic formulas of the *Salmonella* serovars, 7th revision. WHO Collaborating Centre for Reference and research on *Salmonella*. Institut Pasteur, Paris, France.

16. Pouttu, R., T. Puustinen, R. Virkola, J. Hacker, P. Klemm and T. K. Korhonen. 1999. Amino acid residue Ala-62 in the FimH fimbrial adhesin is critical for the adhesiveness of meningitis-associated *Escherichia coli* to collagens. Mol. Microbiol. 31:1747-1757.

17. Purcell, B. K., J. Pruckler and S. Clegg. 1987. Nucleotide sequences of the genes encoding type 1 fimbrial subunits of *Klebsiella pneumoniae* and *Salmonella typhimurium*. J. Bacteriol. 169:5831-5834.

18. Ramphal, R., J. C. Sadoff, M. Pyle and J. D. Silipigni. 1984. Role of pili in the adherence of *Pseudomonas aeruginosa* to injured tracheal epithelium. Infect. Immun. 44:38-40.

19. Rhen, M., P. H. Mäkelä and T. K. Korhonen. 1983. P-fimbriae of *Escherichia coli* and subjected to phase variation. FEMS Microbiol. Lett. 19: 267-276.

20. Rossolini, G. M., P. Muscas, A. Chiesurin and G. Satta. 1993. Analysis of the *Salmonella* fim gene cluster: identification of the new gene encoding a fimbrin-like protein and located downstream from the fimA gene. FEMS Microbiol. Lett. 114:259-266.

21. Tam, J. P. 1994. Immunization with peptide-carrier complexes: traditional and multiple-antigen peptide systems. In Peptide Antigens - A Practical Approach. Ed. G. B. Wisdom. IRLpress, Oxford, U.K.

22. Turner, K. and C. Edwards. 1997. Survival of *Salmonella typhimurium* in aquatic microcosms. In Microbial Response to Stress. Congress publication, Sesimbra, Portugal. March 1997.